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TITLE: Biological Mechanisms of Metastasis Suppression: Which Steps in the Metastatic Cascade are Inhibited by the Metastasis Suppressor Gene BRMS1?

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14. ABSTRACT

The purpose of this research is to determine which steps in the metastatic process are affected by the metastasis suppressor gene, BRMS1. Stable b-Gal expressing MDA-MB-231 and MDA-MB-231/BRMS1 cell lines were used to identify 2 steps in the metastatic process inhibited by BRMS1. It was found that BRMS1-expression (1) reduced the numbers of solitary cells that survive initial arrest in the lung (4 hours) and (2) reduced the numbers of cells that initiate form microscopic lung metastases (4 weeks) (both $p < 0.05$). Both these decreases account for the 80% reduction in lung metastases seen after 8 weeks. In vitro work has shown that anchorage dependence may account of the reduction seen after 4 hours and work is underway to determine factors that may be responsible for the growth inhibition. To determine if BRMS1 suppresses metastatic growth in liver, BRMS1 over-expressing cells and controls were injected via the mesenteric vein to target the liver. No suppression of liver metastases was seen by BRMS1 expression in MDA-MB-435 breast cancer cells. To date these studies have shown no significant difference between the growth rates and vascularity (assessed by H&E and CD31) of MDA-MB-435 ± BRMS1 primary tumors in nude mice. However, a significant lag in initial tumor detection in BRMS1-expressing MDA-MB-435 cells was observed. Furthermore, a significant reduction in the numbers of functional vessels in BRMS1-expressing primary tumors has been shown and work in progress will determine the extent of this reduction.

15. SUBJECT TERMS
Breast cancer, cancer biology, metastasis suppressor genes, in vivo videomicroscopy, animal model, BRMS1, metastasis inhibition

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Table of Contents

Introduction

Despite dramatic improvements in our understanding and treatment of cancer over the past century, almost 1.4 million cases of invasive cancer will be diagnosed in the United States in the coming year and approximately 600,000 cancer deaths will occur. 15% (215,000) of these new cases and 7% (40,000) of all cancer deaths will be from breast cancer, primarily due to metastatic disease {Jemal, 2004 #19}. Metastasis is the spread of cancer from the primary site (e.g., the breast) to other sites throughout the body (frequently brain, bone, liver and lung for breast carcinoma). Fortunately, the metastatic process is inefficient, with only a few metastases developing from millions of cancer cells shed from a primary tumor.

Localized breast cancer is confined to the breast, however one third of all new cases of breast cancer have metastases at the time of diagnosis. Metastases can be defined as either regional or distant. Regional metastatic breast cancer is disease adjacent to the primary tumor, for example, a draining lymph node in the chest. Distant metastases are secondary cancers in remote organs such as the lung or the liver. Survival rates for patients with breast cancer diminish steadily from those with tumors that are discovered and treated when organ-confined, to those patients with regionally metastatic disease, and to those patients with metastases in distant organs. Distant metastases portend certain death for many patients, and may appear many years after apparent successful treatment of the primary tumor due to the presence of clinically unapparent micrometastases at the time of initial therapy. Thus, along with better therapies for treating metastasis, a better understanding of the process is needed to improve survival rates for the majority of patients with metastatic disease.

A novel set of genes has been discovered that can suppress the metastatic ability of a variety of cancer cells {Kauffman, 2003 #6;Welch, 2000 #17;Cromer, 2004 #2;Debies, 2001 #9}. On of these genes, Breast Cancer Metastasis Suppressor 1 (BRMS1) was first identified in the laboratory of Dr. Danny Welch {Seraj, 2000 #18}. Over-expression of the BRMS1 gene in breast carcinoma cells reduces both spontaneous lung metastases (cells seeded from the formation of a primary tumor) as well as experimental lung metastases (cells injected direct by

the tail vein) in nude mice. Although molecular information has been gathered {Samant, 2000 #11;Meehan, 2003 #5}, how the BRMS1 gene leads to metastasis suppression is still largely unknown. We are collaborating with Dr. Welch and are using our ability to quantify sequential steps in hematogenous metastasis in order to answer the following question: ***Which step(s) in the progression of breast cancer metastasis are affected by this metastasis suppressor gene?*** I am using two human mammary carcinoma cell lines, MDA-MB-435 and MDA-MB-231 (highly metastatic in spontaneous and experimental metastasis assays, respectively), and BRMS1 transfectants of the same cell lines (non metastatic to lung) to answer this question.

Body

Task 1 (1-16 months) To compare the growth rate and vascularity of BRMS1-expressing and control primary tumors in the mammary fat pad (m.f.p.) and to compare the ability of the BRMS1-expressing cancer cells to control to arrive at secondary sites (lung and lymph node).

This task is ongoing (90% complete)

As previously reported, I have determined that there is a reduction/suppression in lung metastatic burden at end point (14 weeks) following growth of a mf.p. tumor of MDA-MB-435 cells transfected with BRMS1, relative to parental cells. I have also determined from H&E stained histological sections and from immuno-histochemistry for CD31 (a clinically used immuno-histochemical marker for newly formed endothelial cells) that BRMS1-expressing and control primary tumors have similar total numbers of blood vessels ($p > 0.05$). However, I have found that when numbers of functional vessels are quantified (functional vessels defined as containing 3 or more red cells) there are significantly fewer functional vessels in primary tumors when BRMS1 is over-expressed (Table 1). A reduction in the numbers of functional blood vessels in a primary tumor with BRMS1-overexpressing MDA-MB-435 cells thus may in part be responsible for the reduction in spontaneous lung metastases seen at end point.

Table 1- Vessel counts for primary tumors of parental and BRMS1-expressing MDA-MB-435 mammary fat pad tumors. * = $p < 0.001$, Mann-Whitney Test. 5 high powered fields (200x) were taken per section of primary tumor. All numbers displayed are rounded averages over the number of fields counted.

Cell line	Total number of vessels per high powered field	Number of functional vessels per high powered field
MDA-MB-435	16	11
MDA-MB-435/BRMS1	15	6*

a) Comparison of the total numbers of functional vessels in primary tumors by histological detection of vessels and red blood cells versus observation of function in vivo.

A reduction in the number of functional vessels in BRMS1-expressing primary tumors was seen in histological sections (Table 1); vessels were detected by histological section and functionality was defined by the presence of three or more red blood cells within the vessel. As an alternative method to assess vessel functionality within the tumors, an imaging modality has been used that allows me to measure the entire primary tumor functional vasculature in vivo. Microcomputed tomography (μCT) allows for rapid whole tumor scanning and, together with a clinically used contrast agent (Omnipaque™ ~150 - 200mg), we are able to directly observe the entire functional primary tumor vasculature.

BRMS1-expressing and parental MDA-MB-435 cell lines were injected into the m.f.p. of female mice and primary tumors allowed to develop. Once the primary tumors reached 1.5 cm mean diameter mice were imaged twice. Firstly, live mice were anesthetized and injected with the contrast agent and scanned using the μCT scanner at the Robarts Research Institute. Secondly, the mice were sacrificed and perfused with Microfil™ (a latex compound which is radio-opaque) for high-resolution imaging of functional vasculature of the m.f.p. tumors. Initial live scans have been completed but not analyzed. Ongoing work is to complete the second high-resolution scans, both of which may reveal possible changes in the numbers of functional vessels in these primary tumors.

b) Determining the numbers of BRMS1-expressing and parental MDA-MB-435 breast cancer cells shed to lung from primary tumors.

A task in year 1 was to determine the numbers of cells shed from different sizes of both types of primary tumor. A possible contributor to BRMS1-mediated metastatic suppression may be the inability of cells to effectively leave the primary tumor. To arrive in the lung microenvironment cells must invade out from the primary site (breast) and migrate into a nearby vessel, thus entering into the circulation and being carried to a secondary site (lung). BRMS1-expressing MDA-MB-435 breast cancer cells have shown no difference in invasion and only a small decrease in migration in vitro {Seraj, 2000 #6} when compared to parental cells. Task 1)a) will quantify the functional vasculature of primary tumors and determine if BRMS1-expressing cells form primary tumors with reduced functional vasculature. A reduction in the numbers of functional vessels could possible contribute to a reduction in cells arriving in the lung. Thus, in Task 1)b), cells shed from the primary that arrive in the lung will be directly quantified.

MDA-MB-435 ± BRMS1 cell lines do not contain a marker for detection of single cells (e.g. Green Fluorescent Protein (GFP) or LacZ); therefore, the first step to accomplish this task was to introduce a marker for detection in the lung. LacZ transfection has been attempted but no confirmed LacZ positive clones have been created to date (22 months) due to failure of any clones to maintain growth under selective pressure from hygromycin.

In the meantime, our collaborator has successfully created GFP expressing cell lines for both high BRMS1 expressing and parental MDA-MB-435 cells. Given our on-going collaboration, we will be able to use these detectable cell lines to quantify the numbers of cells shed at different sizes of primary tumor, we will receive these cells shortly (August 2005). The lung will be analyzed for the numbers of cancer cells under a fluorescent microscope and the primary tumors analyzed for percentage necrosis, vascularity (CD31) and proliferation (ki-67)/apoptosis (caspase-3) index.

Task 2 (13-28 months) To assess if there is organ specificity in BRMS1 mediated metastasis suppression. The two organs that will be investigated are lung and liver.

This project is ongoing (85% complete)

Metastases may grow after cells have arrested due to size restriction in the first capillary bed they encounter {Chambers, 2002 #14}. After this arrest, cancer cells may or may not survive, and start to proliferate to form overt metastases, both of which may be organ specific. Welch et al. have preliminary data from a melanoma model in which metastasis suppression due to introduction of a metastasis suppressor gene is organ-specific {Chambers, 2002 #14}. They have found that C8161 melanoma cells that contain a metastasis suppressor gene from chromosome 6 are completely suppressed for metastasis after intra-dermal or intravenous injection. However, after intra-cardiac injection these cells form metastases in bone, which suggests that the metastasis suppressor gene is unable to suppress metastasis in this organ. It is not known whether the BRMS1 gene similarly affects the ability of breast carcinoma cells to form metastases at certain sites. Lung and liver are common sites for human breast cancer metastasis; therefore, determination of whether BRMS1 can suppress metastasis to one or both organs might help to explain organ specific-metastases. The goal of Task 2 is to determine if BRMS1 suppresses liver metastasis as well as lung metastases.

a) Lung metastasis suppressed by BRMS1

As reported previously in the year 1 and 2 reports, cells of the MDA-MB-231 breast carcinoma cell line in which BRMS1 was over-expressed showed an 80% reduction in lung metastatic burden at 8 weeks after tail vein injection (compared with the parental cell line). The introduction of LacZ into both the MDA-MB-231 cell lines (the vector control and the cell line over expressing BRMS1) did not alter the growth kinetics of lung metastases. Thus, BRMS1 has been shown to be able to suppress the formation of lung metastasis of these cells.

b) Liver metastases from injection of MDA-MB-231 and MDA-MB-435 cells

Metastasis suppression thus has been seen in the lung, but it is not known whether this effect of BRMS1 occurs in other organs also. Since the microenvironments in lung and liver differ

considerably, it is reasonable to assess if BRMS1 also inhibits metastasis formation in liver also. Determination if BRMS1 does, or does not, suppress liver metastases may contribute to an understanding of how BRMS1 suppresses the metastatic process.

To first determine if parental MDA-MB-231 breast cancer cells would form liver metastases, MDA-MB-231 cells were injected into the mesenteric vein of nude mice in the following numbers; $5x10^5$, $1x10^6$, and $2.5x10^6$ cells in 200 µl. However, growth of MDA-MB-231 cells in liver has been reported from intra-cardiac injection {Sharp, 2004 #20;Tester, 2004 #21}. MDA-MB-231 cells have not been found to form liver metastases after mesenteric injection. No growth was seen after 13 weeks in any of the mice by gross observation at the time of sacrifice or in analysis of H&E sections by a trained pathologist. After each experiment in vitro plating efficiency[1] was routinely assessed and no significant difference between pre- and post-injection was seen. Thus this model cannot be used to determine the effect of BRMS1 on formation of liver metastases, however, intra-cardiac injection will be used to determine if MDA-MB-231 parental cells will form liver metastases.

To determine if MDA-MB-435 human breast carcinoma cells would form liver metastases, parental MDA-MB-435 cells were injected via the mesenteric vein of mice. Two different cell numbers were chosen ($1x10^6$ and $2.5x10^6$ cells per mouse) and growth of liver metastases was seen in both injection groups. Large metastases developed 4 weeks post injection when $2.5x10^6$ cells were injected and after 8 weeks with the lower cell number. Thus, the MDA-MB-435 breast cancer cells could be used to assess the ability of BRMS1 to inhibit the formation of liver metastases. Subsequently, BRMS1 expressing MDA-MB-435 cells were injected into the liver of nude mice ($1x10^6$ cells per mouse), however no reduction in growth was seen ($n=4$, $p > 0.05$). A follow-up experiment was started with a larger number of animals, but this experiment was ended prematurely as all the animals died due to unexplained interperitoneal bleeding 2 days post surgery. No cause for this could be found however, a possible explanation could be the dissolution of the clot that forms around the injection site in the mesentery resulting in uncontrolled interperitoneal bleeding. Therefore, this experiment is currently being repeated with

[1] Plating efficiency is calculated by plating a known number of cells and counting the formation of colonies, 50% plating efficiency would indicate that half of the cells plated were able to form colonies.

the same number of animals (n=16) to confirm the initial results, results to date have shown which showed no inhibition of liver metastases formation by BRMS1 in MDA-MB-435 cells.

Lack of suppression of liver metastasis by BRMS1 over-expression, if confirmed, could mean that differences between the lung and liver microenvironment may help explain a mechanism for BRMS1 metastasis suppression. Chemokine/chemokine receptor interactions are a possible avenue for investigation that may explain the differences seen in organ specificity. BRMS1 may down regulate the expression of a chemokine receptor which is necessary for the formation of lung but not liver metastases. Flow cytometry will be used to assess the expression levels of chemokine receptor 4, the level of this receptor and its associated chemokine, SDF-1, have been linked to the aggressiveness of breast cancer cells {Kang, 2005 #16}.

Task 3 – (20-36 months) To determine at which step(s) in the metastatic process are inhibited by BRMS1 expression in the formation of liver and lung metastases.

Task 3 is ongoing (85% complete)

Identification of the step(s) in the metastatic cascade at which BRMS1 suppresses metastases may help to determine step(s) in breast cancer metastasis that are inherently sensitive to inhibition, thus providing insights into possible directions for new research into anti-metastatic therapy.

The growth kinetics (survival and growth curves) of MDA-MB-231 cells in lung were detailed in the year 2 report and were the basis for the time points selected in determining at which step(s) in the metastatic cascade BRMS1 might inhibit lung metastasis formation. The time points initially chosen were 4 hours, 1 day, 2 days, 1 week, and 4 weeks post-injection. Subsequent to completion of this work, further time points were added at the beginning of this time sequence (Figure 1) to narrow down the time point at which the reduction in the numbers of solitary cells was occurring. These time points were 5 min, 30 min, 1 hour and 2 hours post-injection.

a) Determination of where in the metastatic process BRMS1 inhibits liver metastases

Since no suppression of liver metastases by BRMS1 in MDA-MB-435 cells has been seen 2)b), it is not possible to determine the step at which BRMS1 inhibits the process. Ongoing work will determine if there is any suppression by BRMS1 in a model of breast cancer liver metastasis from direct intravenous intra-cardiac or mesenteric injection.

b) Determination of the step(s) in the metastatic process where BRMS1 inhibits lung metastases

To determine if BRMS1 suppresses early events in metastasis, the proportions of single cells and micrometastases were quantified at the times indicated in Table 2 in both BRMS1-overexpressing and parental MDA-MB-231 cells. Five minutes post-injection, 95% of the initial cell number of both cell lines were present in the lung. The 5% of the total number of cells not present in the lung were assumed to have found a way through the lung microvasculature (i.e. through shunts), or were lost to cell death. Other organs (brain, spleen, liver, ovaries, kidneys) were stained for β-gal activity and no staining was observed under a dissecting microscope. No metastases or β-gal staining were observed in any other organs other than lung after 8 weeks; these results are shown in Figure 1.

Table 2 – Results from examination of lungs after tail vein injection with parental MDA-MB-231 breast cancer cells. The time course shown was used for all experiments. Percentages shown are determined by 'cell accounting' {Luzzi, 1998 #13}. $1x10^6$ cells in 200µl of Hank Buffered Salt Solution were injected per mouse. Micrometastases here are defined here as clusters of cells with diameter within 50 –1000µm. At 8 weeks all mice were sacrificed due to large lung metastatic burden. See **Figure 1** for quantitative results.

Time Point	% of parental cells remaining as solitary cells	Description of Results
5 minutes	~95 %	Solitary cells present after initial arrest in lung
30 minutes	~80%	
1 hour	~60%	
2 hours	~50%	
4 hours	~10%	
1 day	~10%	
2 days	~10%	

1 week	~10%	Initial detection of micrometastases from a subset of solitary cells
4 weeks	~5%	Maintenance of growth of micrometastases (subset of micrometastases)
8 weeks	~0.001%	Large overt vascularized metastases in lungs (~100 surface metastases)

In the lung, a gradual reduction in tumor cell numbers in both cell lines (231-BRMS1 and parental) was seen until 4 hours post-injection, with approximately 10% of the parental cell line remaining. A significant reduction in numbers of BRMS1 over-expressing cells compared to the parental cell line (~40% reduction, $p < 0.05$) was seen at all time points after 4 hours (Figure 1). Thus, BRMS1 over-expression effects the initial arrest of MDA-MB-231 breast cancer cells in lung.

At 4 weeks post-injection, significantly fewer BRMS1 over-expressing MDA-MB-231 cells initiated growth to form micrometastases in mice, when compared to parental cells (~50% reduction, $p < 0.05$), Figure 2A, even when correcting for the pool of cells from which micrometastases could arise, Figure 2B. Correcting for the reduced pool of BRMS1-expressing cells involved standardizing each cell line to the numbers of surviving solitary cells at 4 hours. However, there was no difference in either the median size of micrometastases, Figure 2C, or in the range in size, Figure 2D, of the micrometastases seen. Thus, BRMS1-expression effects the initiation of growth of micrometastases of MDA-MB-231 cells in lung.

The reduced ability of BRMS1 over-expressing cells to survive initial arrest in the lung when compared to controls indicates that BRMS1-expressing cells may be less able to survive insult by factors released at the time of arrest (e.g. nitric oxide; NO). The inhibition of growth into micrometastases of BRMS1 over-expressing cells suggests that these clusters of cells may also be sensitive to factors released in the lung and/or initiate growth in an anchorage independent environment. Work is ongoing to determine the levels of proliferation (Ki-67) and apoptosis (caspase-3) at the time of initial arrest of cells and in metastases (at 4 weeks post-injection). This work will help clarify whether cell over-expressing BRMS1 are more likely to undergo apoptosis when in the lung microvasculature.

c) Analysis of early time points by IVVM

As mentioned in the year 2 annual report, intravital video microscopy (IVVM) was used to visualize cells labeled with a transient cytoplasmic marker (CFMDA™). The goal of this work was to directly observe cancer cells within the microvasculature to determine if BRMS1 changes the morphology of breast cancer cells. Both BRMS1-expressing and control MDA-MB-231 cells were visualized up 2 hours post-injection in the microvasculature. BRMS1 expressing cells were observed to remain rounded, and blocked blood flow, (Figure 3) in the microvasculature whereas parental control cells were observed to flatten out (Figure 4) onto a vessel, and allow blood flow pass by, during the time observed. This finding leads to the following hypothesis: BRMS1 over-expression reduces the ability of MDA-MB-231 cells to adhere to the vessel wall, thus not allowing them to flatten out along the vessel wall. This lack of adherence may explain the reduction in number of BRMS1 over-expressing cells seen 4 hours post injection when compared to controls. Parental MDA-MB-231 cells that adhere more readily may therefore be better able to survive insult by factors released at the time of arrest.

Work is ongoing to determine if BRMS1 cells are more sensitive to factors released from the lung microvasculature at the initial time of injection. Work by Orr et al. {Qiu, 2003 #11} has shown that NO is released when cells initially arrest in both lung and liver microvasculature. This work will use SIN-1 (a producer of NO) in vitro and L-NAME (an inhibitor of NO) in vivo to determine if BRMS1 over-expressing cells are more sensitive to NO. Thus, this work will hopefully determine a molecular mechanism for BRMS1-mediated lung metastasis suppression, knowledge of a putative molecular pathway involved may help to identify possible novel therapies for metastasis suppression.

d) Anchorage dependence

It has been shown that adhesion is necessary for cancer cells to successfully form metastases in a secondary site {Glinskii, 2005 #15}. To determine if BRMS1 over-expression reduces the ability of MDA-MB-231 breast cancer cells to form foci in an anchorage independent assay. A difference in anchorage independent growth may thus explain why fewer BRMS1 over-expressing cells form micrometastases in vivo 4 weeks post injection.

An in vitro assay was used to determine if anchorage-independent survival and growth was reduced when BRMS1 is over-expressed. Briefly, this involved coating tissue culture plates with a 1% layer of Agarose (hard agar), then layering a mixture of 0.6% agarose (soft agar) combined with a suspension of cells ($1x10^4$ cells total per plate). Plates were allowed to solidify and media was added and changed every 3 days throughout the duration of the experiment (15 days). BRMS1-expressing MDA-MB-231 cells showed a 37% reduction in numbers of colonies formed and decreased median size of colony by 22% when compared to controls (Table 3, $p < 0.05$), however there was no difference in either the mean size or maximum size of colony seen. Counts were determined by capturing images on an inverted Nikon © microscope and processing the imaging using ImageJ© (National Institutes of Health) software to calculate the Feret's diameter {Dolapchieva, 2004 #12} of separate colonies. Four images per $60mm^2$ plate were captured and nine plates per cell line were used in this assay. The decreased ability of BRMS1-expressing cells to form colonies in soft agar may contribute to what is seen in vivo, where an ~80% reduction in metastatic ability was seen.

Table 3 - Anchorage Independent Growth and Survival Assay. A description of this assay is given in section 3)c) Quantification was performed by collecting images on a Nikon © microscope and analysed using ImageJ™ (NIH) software. A Field of View (FOV) here is defined as a single picture with approximate dimensions of 2,300 x 3,000 µm. Statistics were calcuated using Mann Whitney Test, * = $p < 0.05$

	Mean Size (µm) ± SE	**Max Size (µm)**	**Average # Colonies per FOV**	**Median Size (µm)**
231	107.7 ± 7.3	1822	46	61.02
231/BRMS1	105.6 ± 11.3	1768	29*	47.45*

Thus, the reduction in plating efficiency of BRMS1 expressing cells in an anchorage independent environment may help to partially explain the reduction in number of BRMS1-expressing micrometastases seen in vivo 4 weeks post injection and explain the lack of difference in mean and maximum size seen in this in vitro assay.

Key Research Accomplishments

- Showed that there was no difference in the number of vessels in primary breast tumors with or without BRMS1 when analyzed by immunohistochemistry (CD31) or H&E. However, there is a small but significant reduction in the number of functional vessels (a functional vessel is defined here as a vessel containing 3 or more red blood cells, i.e. perfused) in primary tumors expressing BRMS1 when compared to controls.
- Started using a microcomputed tomography system to image whole mice with tumors to determine the numbers of functional vessels in BRMS1-expressing and control primary tumors in mice and compare theses values with those seen in histological sections.
- Used IVVM to determine that when BRMS1 is over-expressed cancer cells appear to remain rounded and non-adherent to the vessel wall, whereas control cells flatten out along the vessel wall.
- Used in vitro assays to determine that MDA-MB-231 breast cancer cells have a reduced ability to grow in an anchorage-independent manner when BRMS1 is over-expressed.
- Determined that MDA-MB-231 cells are unable to grow as liver metastases from mesenteric vein injection over a range of cell numbers injected. In contrast MDA-MB-231 cells are able to form lung metastases.
- Determined in a preliminary experiment that although parental MDA-MB-435 cells do form liver metastases, BRMS1 does not inhibit the ability of these cells to form liver metastases, when injected with two cell numbers $1x10^6$ and $2.5x10^6$/cells per mouse.
- Determined that BRMS1 over-expressing MDA-MB-231 cells show reduced ability to survive after initial arrest in the lung.
- Determined that BRMS1 over-expression reduces the number of solitary breast cancer cells that initiate growth to form micrometastases in lung.
- These studies have shown that effects on initial arrest and initiation of growth are critical steps in BRMS1 mediated metastasis suppression
- Presented an abstract/poster at the 2005 Era of Hope conference (June 8-11, 2005) detailing the steps at which BRMS1 has its effect on the metastatic process.

Reportable outcomes

- Received a Graduate Tuition Scholarship from the Faculty of Graduate Studies, University of Western Ontario (UWO), September 2002 - September 2005.
- Presented this work at; The Experimental Oncology Seminar Series (UWO), January 2005, The A.C. Burton Day Lecture, March 23, 2005, and The Second Annual Oncology Research Day, London Regional Cancer Program, London Health Sciences Centre.
- Showed that there was no difference in the number of vessels in primary breast tumors with or without BRMS1 when analyzing by immunohistochemistry (CD31) or H&E. However, there was a difference when counting the number of functional vessels (a functional vessel here is defined as containing 3 or more red blood cells).
- Started using a microcomputed tomography system to compare the numbers of functional vessels in BRMS1-expressing and control primary tumors with those determined by analysis of histological sections.
- Determined by IVVM that when BRMS1 is over-expressed cancer cells appear to remain rounded in the mouse microvasculature whereas controls flatten out along the vessel wall.
- Determined that BRMS1 over-expression reduces (~37%) the ability of breast cancer cells to grow in an anchorage independent in vitro growth system.
- Determined that parental MDA-MB-231 cells, when injected over a range of $5x10^5$ to $2.5x10^6$ cells per mouse, are unable to form liver metastases, however, parental MDA-MB-435 do form liver metastases when injected at $1x10^6$ or $2.5x10^6$ cells per mouse.
- Determined that BRMS1 does not inhibit the ability of MDA-MB-435 cells to form liver metastases, when injected at $1x10^6$ or $2.5x10^6$ cells per mouse.
- Confirmed that the numbers of BRMS1 over-expressing cells remaining in lung are reduced by ~40% 4 hours post injection, when compared to controls.
- Determined that BRMS1-expressing cells form decreased numbers of microscopic metastases at 4 weeks. When this data is corrected for the reduced pool of cells from which microscopic metastases could arise, a ~43% reduction in BRMS1-expressing MDA-MB-231 cells that initiate growth to form microscopic metastases is seen.
- Attended and presented a poster at the Era of Hope 2005 meeting in Philadelphia, PA (June 8-11, 2005).

Conclusions

The reduction in spontaneous lung metastasis burden by BRMS1 over-expression may in part be due to the reduction in the number of vessels able to carry cancer cells from the primary tumor to secondary sites. To address this question a novel mouse imaging technique (Microcomputed tomography, μCT) is now being used to assess the total number of functional vessels in live mice. Previously reported work quantified the total numbers of vessels in primary tumors by either immunohistochemistry for CD31 or analysis of H&E sections and demonstrated that BRMS1-overexpression does not change the total number of vessels. However, quantification of the number of functional vessels (defined by 3 or more red blood cells) revealed a significant reduction in BRMS1-overexpressing MDA-MB-435 cells. The results of this new work with μCT will quantify the number of perfused vessels in mammary fat pad tumors and compare them to those determine from histological sections. Thus we will be able to determine whether BRMS1 mediated reduction in lung metastasis is partially due to reduced functional primary tumor vasculature.

A reduction in functional vasculature of BRMS1-expressing primary tumors may result in reduced numbers of cancer cells arriving in lung. To determine the numbers of cells arriving in the lung over time it is necessary to label both cell lines (BRMS1-expressing and parental MDA-MB-435) with a marker for detection. LacZ has been used to label BRMS1-expressing and parental MDA-MB-231 breast cancer cells, however to date no MDA-MB-435 clones have maintained growth under selective pressure. Our collaborator has successfully created GFP-expressing clones of BRMS1-expressing and parental MDA-MB-435 cells and we thus will be able to use these detectable cell lines to quantify the numbers of cells shed to lung from primary tumors.

Previous work reported in Year 2 has shown that there is a difference in the initial survival of MDA-MB-231 and BRMS1-expressing MDA-MB-231 cells in the lung. The work presented in this report has refined the kinetics of BRMS1 mediated lung metastasis suppression and show a reduction in the numbers of cells persisting in the lung 4 hours after injection. Thus, BRMS1 over-expression has been shown to reduce survival of breast cancer cells in lung after initial arrest. Work is ongoing to determine the rates of apoptosis at 4 hours, which will allow

determination of whether BRMS1 cells are more likely to undergo apoptosis when in the lung microvasculature.

Along with this initial reduction in cell number, there is a reduction in the number of micrometastases formed 4 weeks post-injection. Thus, BRMS1 over-expression has been shown to limit the growth of micrometastases from the population of solitary cells that survive the initial arrest. Work is ongoing to determine the rates of proliferation and apoptosis at 4 weeks post injection, which will allow determination of whether BRMS1 micrometastases are more likely to undergo apoptosis. Together, the difference in numbers of solitary cells at 4 hours and the reduced ability to initiate growth of microscopic metastases after 4 weeks can account for the 80% suppression of metastasis seen at end point.

The initial reduction in numbers BRMS1-expressing cells maybe due to changes in way they arrest and/or adhere to the microvasculature in the first 4 hours after injection. IVVM was used to investigate early time points after injection of cells. This work has shown that over-expression of BRMS1 changes the appearance of cells within the microvasculature. BRMS1 cells appear rounded, whereas control cells flatten along vessel walls. It was hypothesized that this difference was due to a lack of adherence to the vessel wall and was investigated further in an in vitro assay for anchorage-independent growth. A ~40% reduction was found in the ability of BRMS1-expressing MDA-MB-231 cells to grow in soft agar (anchorage independent medium) when compared to controls. The in vivo observations and in vitro results suggest that the inability of BRMS1-expressing cells to grow in anchorage independent environment may explain the reduction in growth of BRMS1-expressing cells seen in lung 4 weeks post injection.

These studies have shown that effects on initial arrest (decreased cell survival) and initiation of growth are critical steps in BRMS1 mediated metastasis suppression. Furthermore, results from in vitro work have shown that a reduction in growth in an anchorage independent medium and may explain the reduced numbers of micrometastases seen at 4 weeks. Biological information on how the loss of BRMS1 can promote breast cancer metastasis and at what step in the metastatic process may provide insight into possible directions for the development of novel anti-metastatic therapeutic strategies.

References

Appendices

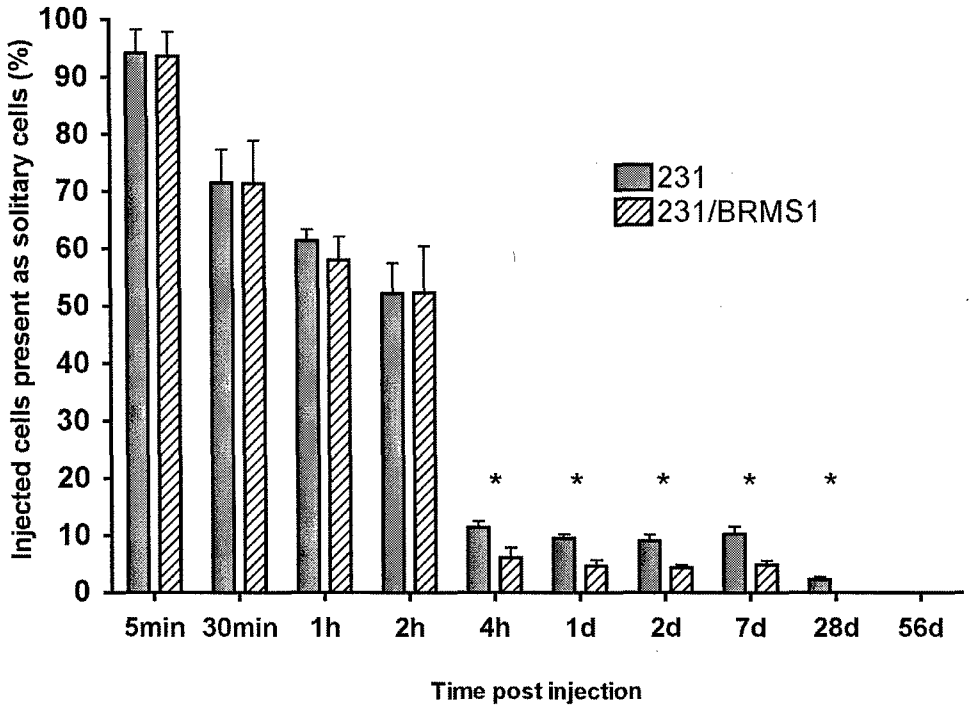

Figure 1 - Number of solitary cells present in lung, after injection of $1x10^6$ MDA-MB-231 ± BRMS1 breast cancer cells into the lateral tail vain of female athymic mice. Cells were suspended in 200µl Hanks Buffered Salt Solution. Percentage of cells was calculated using reference microsphere for each time point (as described {Luzzi, 1998 #13}), at least 4 mice were used per time point. * = $p < 0.05$ as determined by non-paired t-test.

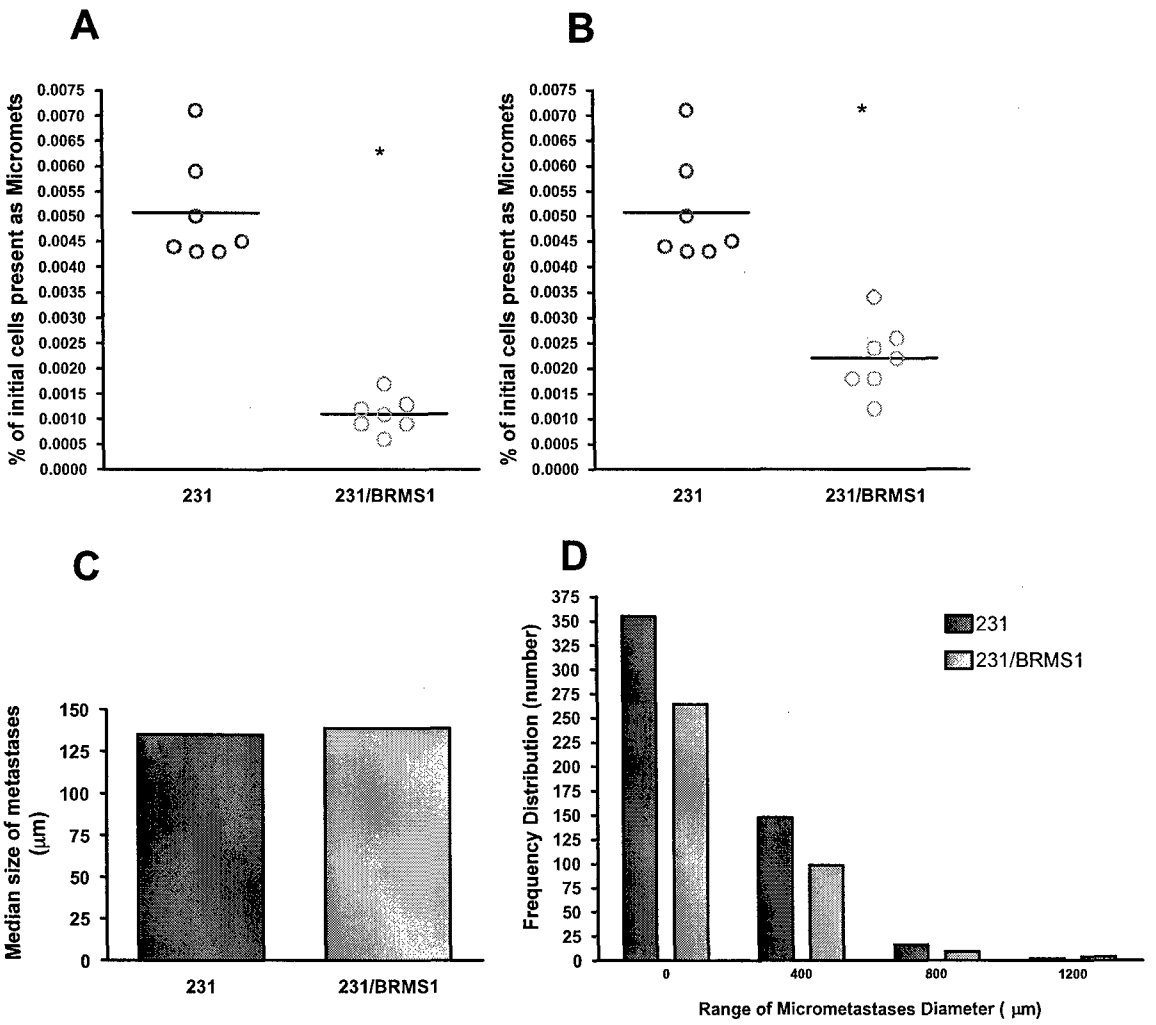

Figure 2 – A. Percentage of initial cells present at 4 weeks as lung metastases (greater than 50μm in diameter). **B.** Percentage of cells was calculated based on the number of surface metastases counted at 4 weeks divided by the initial number of cells injected as in **Figure 1**, # surface lung metastases/10^6. **C.** No significant difference in median diameter of micrometastases at 4 weeks post injection, $p > 0.05$. **D.** Histogram of all diameters of 4 weeks micrometastases. Bin sizes for the histogram was taken to be 400 mm, x-axis shows the bin center.

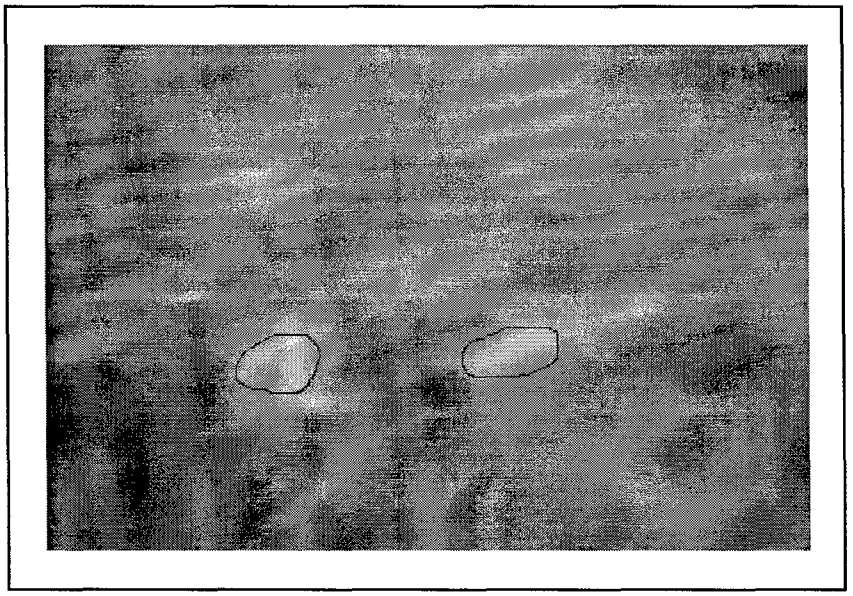

Figure 3 - MDA-MB-231/BRMS1

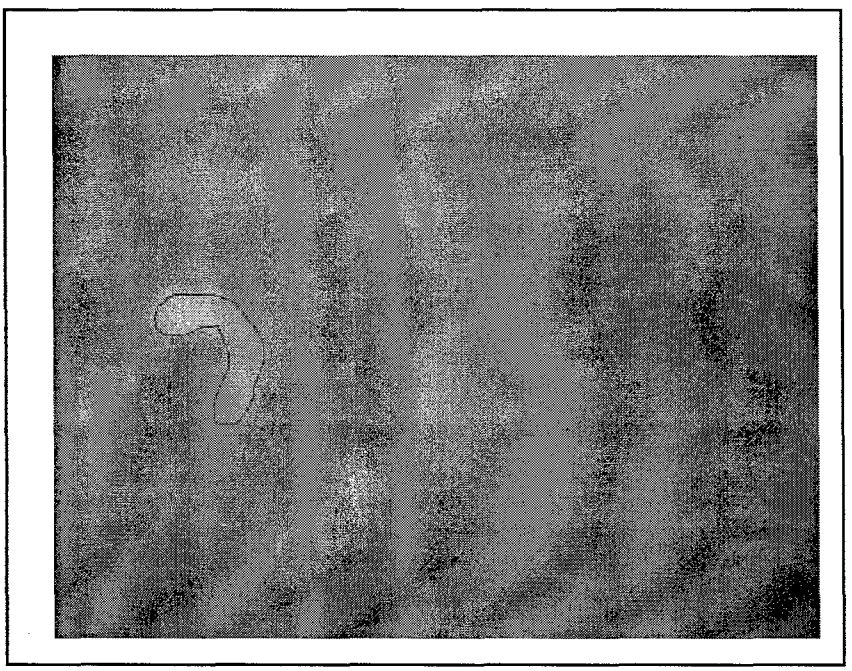

Figure 4 - MDA-MB-231